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MAIN REPORT:

ABSTRACT:

We have isolated a variant form of the estrogen receptor (ER) from breast hyperplasias which displays a 100-fold increase in estrogen sensitivity. We have found this variant to be present in more than 20% of proliferative hyperplasias and also in the normal breast tissue adjacent to the hyperplasia. The research funded by this grant has focused on the function of this super-active ER variant in breast cancer, as well as the factors which regulate its expression in breast cancer cells as potential targets for clinical therapy.

In transient transfection assays, the variant ER is activated by estrogen at much lower concentrations as compared to the wild-type, and forced expression of the ER variant in breast cancer cell lines increases cell proliferation. Expression of a variant ER which confers a proliferative advantage as well as a heightened sensitivity to estrogen might predispose breast hyperplasias to malignant progression. We are currently investigating the mechanism which confers this increased activity to the ER variant.

Regulation of the expression of both the wild-type as well as the variant ER at the transcriptional level is also a potential target for clinical therapy. For this reason we have begun studies of the transcriptional factors which regulate activity of the ER gene promoter. Transient transfection studies in a panel of breast cancer cell lines indicate that the basal promoter of the ER gene lies within the first 245 bp of the 5' flanking region of the gene, and displays unusually high transcriptional activity in transient transfection assays, and the regulatory elements within this region are currently being investigated.

INTRODUCTION:

In vivo and *in vitro* studies of the mitogenic effects of estrogens on various breast cancer cell lines grown in culture, as well as in ovariectomized nude mice has been well documented (6, 8). We have recently isolated a variant of the estrogen receptor (ER)

which displays a 100-fold heightened sensitivity to estrogens. This ER variant is expressed in approximately 20% of proliferative breast hyperplasias tested. We hypothesize that the study of this "super-active" ER variant will provide insight into possibly one of the earliest events in the progression of breast cancer, eventually providing a new target for prevention of this disease for some women. In this fellowship, I propose to address this hypothesis with the following Specific Aims:

1. To study the function of a novel "super-active" ER variant using estrogen-responsive mammalian expression vector systems. (months 1-6)
2. To generate stable breast cancer cell transfectants overexpressing either wild-type ER or the hypersensitive ER for evaluation of altered hormonal proliferative and growth response. (months 6-24)
3. To use the "super-active" ER variant protein as a sensitive tool to search for receptor interacting proteins/coactivators that mediate or help facilitate ER transcriptional activity in human breast cancer cells. (months 18-36)

BODY:

I have made excellent progress in this first year of my investigation focusing predominantly upon Specific Aims 1 and 2 and am on schedule in the grant. I have initiated studies into the functional properties of the ER variant by first generating stably transfected MCF-7 breast cancer cell lines that overexpress either the variant ER or wild-type ER under the control of a constitutive cytomegalovirus promoter. Growth curve studies of these stable transfectants comparing proliferative response at varying concentrations of estradiol from 1×10^{-12} to 1×10^{-9} (Fig. 1) suggest that expression of the ER variant confers a growth advantage to cells at lower concentrations of estradiol (compare panels B and C with panels E and F). Cells were plated at a density of 2×10^4 in media containing 10% charcoal-stripped, estrogen-free fetal calf serum and were either left untreated, or treated with varying estradiol concentrations. The medium was replaced every 48h and the cells were harvested and counted on days 2, 4, 6, and 8.

In the next year I will continue using the cells stably transfected with the variant ER for studies which investigate the biological properties conferred by expression of the ER variant. I plan on continuing the proliferation assays in soft agar experiments, as well as using the stably transfected cells to study DNA and hormone binding properties of the variant ER by Scatchard and gel-retardation assays. By the end of the upcoming year I plan on studying athymic nude mice injected with the stable transfectant cell lines overexpressing the variant ER.

Transactivation studies (Fig. 2) in MDA-MB-231 cells transiently transfected with the variant or wild-type ER and a vitellogenin ERE-tk-luciferase reporter construct suggest that the increase in proliferation exhibited when the ER variant is overexpressed in breast cancer cells may be due to an increase in estrogen sensitivity. In this study, we found that the variant had an $EC_{50} = 10^{-12}$, while the wild-type had an $EC_{50} = 10^{-10}$. Briefly, cells were cotransfected with 1 ug of the reporter plasmid and 15 ng of ER expression vector, either Var or WT. Cells were also cotransfected with 200 ng of a β-galactosidase expression vector, which served as a control for transfection efficiency. After a 12 h transfection period, cells were treated for 48h with concentrations of estradiol from 1×10^{-13} to 3×10^{-9}. Luciferase activity was then determined relative to vector alone, control transfected cells, and corrected for β-galactosidase activity. Transfections were performed in triplicate.

To determine the relationship between the increase in estrogen sensitivity and the increase in proliferation, we are testing whether transactivation of natural ER-responsive promoters such as that of the progesterone receptor gene is increased at lower concentrations of estradiol when presented with the ER variant in transient transfection assays (Specific Aim 1). We are currently subcloning varying promoter fragments of the progesterone receptor promoter into the luciferase reporter vector pGL3-Basic (Promega, Madison, Wi), and will be able to report the results of these studies in next year's report. If the progesterone receptor promoter appears to be a useful reporter for activity of the

ER variant, I will then use it in transient cotransfection assays with both the "super-active" ER variant and the wild-type ER, as well as known coactivators of the steroid receptor family. In the final year of this fellowship, I plan on using the variant ER protein as a tool to search for receptor interacting proteins/coactivators. These set of experiments should provide a great deal of insight into the mechanism(s) by which the variant ER confers a proliferative advantage to those cells in which it is overexpressed.

Since I am interested in the regulation of this ER variant as a potential target in breast cancer therapy, I have also begun studies investigating the factors which regulate the expression of both the ER variant as well as the wild-type ER in breast cancer cells. These studies were not originally included, however the direction of my investigations has lead me to include this additional Aim. Studies have shown that elevated levels of ER in benign breast epithelium is a risk factor for progression to invasive breast cancer (7). ER expression has been shown to be regulated at the mRNA level both in human breast cancer cell lines and in tumors (1, 5, 9, 10, 13). This level of regulation has been shown, at least in cell lines (13), to be at the transcriptional level. For this reason, I am also pursuing studies of the transcription factors which might regulate both wild-type and variant ER expression.

For these studies, I have first created a series of ER 5' promoter deletion fragments that have been subcloned into the luciferase reporter vector pGL3-Basic (Promega, Madison, Wi) (Fig 3A). These ER promoter reporter constructs were designed to include elements that have been reported in the literature to contain potential regulatory or enhancer activity. These elements include the enhancer ER-EHO at -3778 bp first reported by Tang, *et al* (11) within the -4100 fragment(Fig 3A fragment F), three ½ EREs in the -1000 fragment (Fig 3A, fragment D (12)), and in the -245 fragment the basal promoter region first described by this lab (2) (Fig 3A, fragment B). All of the fragments were designed to include the binding sites in the 5' untranslated region of the ER gene for

the factor termed ERF-1, which some studies have suggested might have a regulatory role in ER transcription (3).

All of these reporter constructs were transiently transfected into a panel of ER-positive breast cancer cell lines using the calcium phosphate precipitation method (4) at a concentration of 500 ng, and patterns of promoter activity were evaluated (Fig 3B). Briefly, cells were plated at a concentration of 2 x 10^5 24h prior to transfection, and reporter constructs were cotransfected with 100 ng of a β-galactosidase expression vector, which served as a control for transfection efficiency. Promoter activity was determined as described above. We looked for patterns of activity, and subjected the results to rigorous statistical analysis to compensate for the differences that are inherent in transfection assays when comparing cell lines. The results of these experiments are presented in log form for statistical purposes.

I was unable to detect any statistically significant ($p<0.3$) increase in activity with the larger fragments of the ER promoter as compared to the basal promoter fragment (compare fragments C,D,E, and F with fragment B, Fig 3B). I have therefore focused my studies of the regulation of the ER promoter to the region between -245 bp and +212 bp. I plan to create more 5' promoter deletion reporter constructs to further define the region responsible for the high level of transcriptional activity exhibited in the transient transfection assays, and then create linker-scanner mutations to determine the putative elements within this region. Binding by specific factors will be determined by mobility supershift assays, and biological relevance in regulation of ER promoter activity will be determined by forced expression in co-transient transfection assays with the ER promoter -245 bp reporter.

CONCLUSION:

As is evidenced by this year's report, I have made significant progress toward my goal. I anticipate that I will successfully complete all of my stated Specific Aims by the end of Year 3 of this proposal. I will continue in my efforts at examining the mechanism(s) as well as the physiological effects involved in the ER variant's heightened sensitivity to estrogen, as well as investigating those factors which regulate not only the expression of the "super-active" ER variant, but of the wild-type ER as well. Finally, once I have determined some of the regulating factors of both expression and function, I will attempt to determine their usefulness as targets in therapy for some women with breast disease.

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FIGURE LEGENDS:

Figure 1: Growth curves of stable MCF-7 transfectants in response to increasing concentrations of estradiol in the media. Cells were either left untreated (■), or treated with the indicated estradiol concentrations : 1 x 10^{-12} (●), 1 x 10^{-11} (▲), or 1 x 10^{-9} (◆). Cells were stably transfected with: **A**: untransfected; **B and C**: variant ER; **D**: vector only; **E and F**: wild-type ER.

Figure 2: Transactivation assay of MDA-MB-231 cells transiently transfected with ER expression vectors, either the variant (Var) or wild-type (WT), with estrogen concentrations ranging from 1 x 10^{-13} to 3 x 10^{-9}. (◆) = variant ER ; (■) = wild-type ER.

Figure 3: ER 5' promoter deletion reporter transfection assays. A. ER 5' promoter deletion fragments were subcloned into the luciferase reporter vector pGL3-Basic. B. ER promoter activity assays using transient transfection assays of ER 5' promoter deletion reporter constructs into a panel of ER-positive breast cancer cell lines (MCF-7, T47D, and ZR75-1).

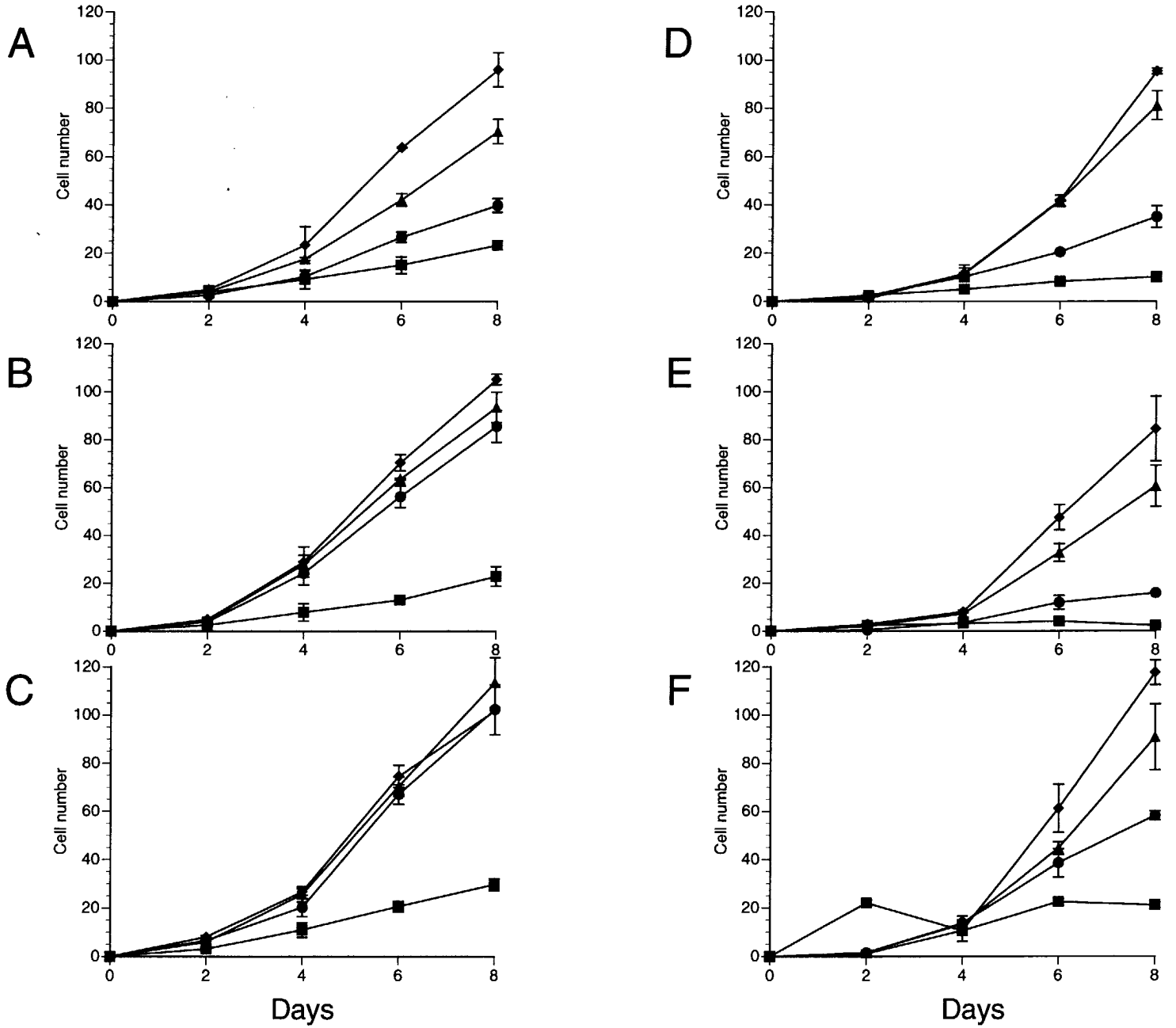
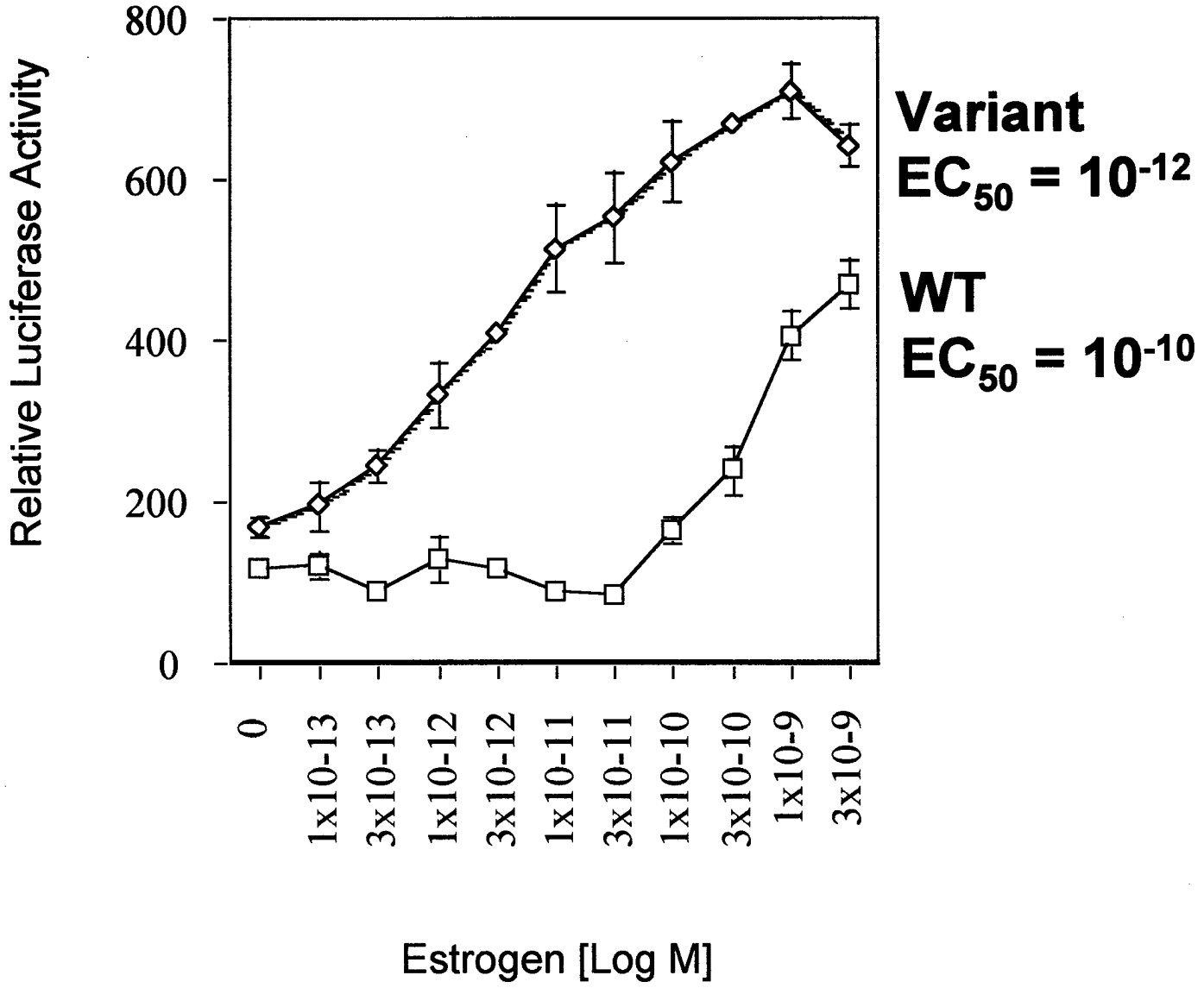


Figure 1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1x10-10
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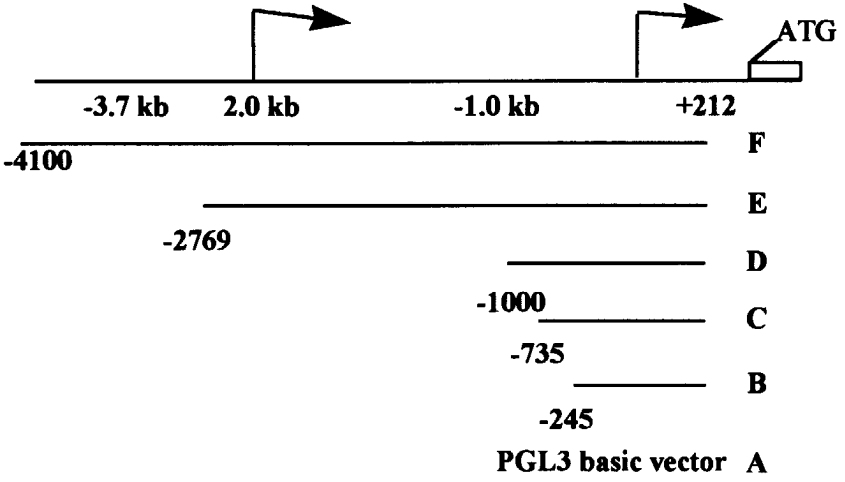
Estrogen [Log M]
Variant
EC50 = 10-12
WT
EC50 = 10-10

Figure 2

A.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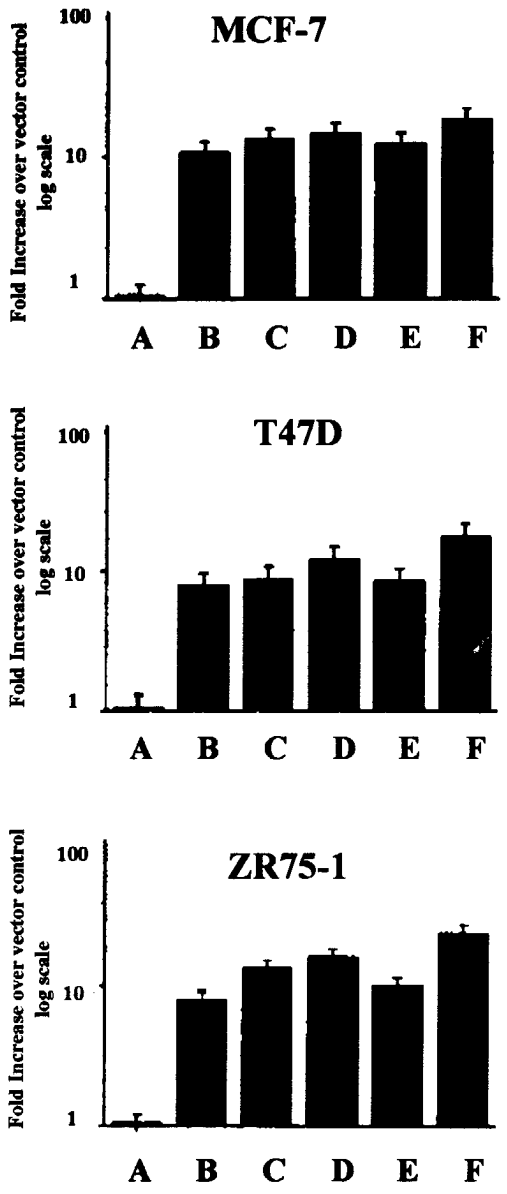
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Figure 3